Experimental focal cerebral ischemia

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Chapter 6

Dopamine improves murine microglial cell survival after hypothermia-rewarming injury partially by a receptor-mediated mechanism

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ABSTRACT

Background: Reducing body temperature by targeted temperature management has been shown to be cytoprotective in various pre-clinical models of organ damage, including the brain. However, rewarming after systemic hypothermia may be accompanied by major complications. Thus, the potential clinical benefits of hypothermia call for stringent measures against a post-hypothermic rewarming insult. Our previous studies have revealed a strong association between hypothermia (both in hibernators and forced induction in rodents) and the endogenous hydrogen sulfide (H_2S) signalling cascade. Moreover, dopamine was found to maintain expression of H_2S synthesising enzymes (CBS, 3-MST and CSE) in smooth muscle cells undergoing hypothermia-rewarming thus rescuing the cells from injury. Here we set out to investigate putative effects of dopamine in murine microglial (BV2) cells against this injury and to decipher potential underlying mechanisms.

Materials & Methods: BV2 cells were subjected to hypothermia (24 h)-rewarming (2 h) injury. Dopamine was exogenously administered with or without relevant inhibitors (haldol, vanoxerine, fluphenazine) throughout the insult. Cell viability was assessed using neutral red assay. Levels of CBS and 3-MST were quantified with western blotting.

Results: Hypothermia-rewarming injury significantly reduced cell viability and expression of CBS and 3-MST. Dopamine treatment concentration-dependently protected BV2 cells from this injury, the effect being abrogated by fluphenazine but not by haldol or vanoxerine, signifying a receptor-mediated action. Moreover, induction of cAMP in these cells mimicked the protection of dopamine. Furthermore, dopamine upregulated CBS and 3-MST expression in BV2 cells that underwent hypothermia and rewarming.

Conclusion: Dopamine rescued BV2 cells from hypothermia-rearming injury by a D1 type receptor-mediated action, likely by the upregulation of the levels of CBS and 3-MST.

INTRODUCTION

The controlled lowering of body temperature to levels of 32-33 ^{o}C, nowadays referred to as targeted temperature management (TTM), has been advocated as a measure against brain disorders in various pre-clinical models of central nervous system (CNS) injury, including cerebral ischemia, traumatic brain injury and spinal cord injury (1-6). The promising results found in these models have sparked the investigation of TTM in clinical trials for CNS disorders such as traumatic brain injury and ischemic stroke (2,7,8). Apart from its therapeutic use in such neurological settings, cooling and rewarming have since long been applied in other clinical situations such as organ transplantation, cardiac arrest and sepsis (9,10). Nevertheless, TTM as a part of clinical management in such disorders may also convey deleterious effects, particularly during and after the rewarming phase (11). Rewarming related cell damage is primarily attributed to an increased intracellular Ca^{2+} influx and production of reactive oxygen species (ROS) (12-14), which in turn may activate resident tissue macrophages and thereby initiate a cascade of events involving apoptosis, necrosis and inflammation. It is therefore essential to counteract these adverse effects of rewarming-induced injury.

Hibernating animals undergo repeated cycles of reduced core body temperature and metabolism (torpor) interspersed with bouts of complete reversal of the body temperature and metabolism to physiological levels (arousals). While these cycles closely resemble hypothermia-rewarming phases, they do not result in overt damage to any organ including the brain. Previous research suggests that induction of endogenous H_2S production constitutes a major mechanism in hibernators that mitigates ROS associated organ damage during arousals (12,15).

Dopamine may represent a compound capable of mobilizing the endogenous H_2S synthesis and protect organs from hypothermia and rewarming damage. Yard et al. (2004) observed the rescuing effects of dopamine in HEK cells exposed to hypothermic injury, which involved ROS scavenging. In addition, dopamine has been demonstrated to alleviate Ca^{2+} depletion in hypothermic human umbilical venous endothelial cells (HUVECs) (16) and to induce hemeoxygenase-1 expression in normothermic HUVECs (17). Interestingly, previous results from our group showed administration of dopamine to alleviate production of ROS and maintain endogenous H_2S production in models of hypothermia and rewarming, both *in vitro* (Talaei et al., 2011) and *in vivo* (18). Moreover, protection is associated with the maintenance of expression of H_2S synthesizing enzymes, including cystathione β-synthase (CBS) (12), 3-mercaptopyruvate sulfurtransferase (3-MST), and cystathione γ–lyase (CSE) (18). Notably, in cell models, dopamine protection seems to be conveyed by the cellular uptake of dopamine (12).

Given the protective effects of dopamine in various non-CNS cells, we here set out to explore dopamine's efficacy in protecting microglial cells from hypothermia and rewarming induced damage. Microglia represents the resident macrophages in the brain and get activated upon various pathological insults. We therefore employed a widely used microglial cell line (BV2 cells) to study whether dopamine rescues these cells from hypothermia and rewarming-induced injury and elucidate potential mechanisms of protection.

MATERIALS & METHODS

Cell culture

Murine BV2 microglia cells were cultured in a T75 flask in DMEM (containing 4.5 g/l glucose, 584.0 mg/l L-glutamine, GIBCO) supplemented with 5% FBS, 1% penicillin-streptomycin at 37°C in a humidified incubator supplied with 5% CO_2 atmosphere. The cells were generously procured from the Department of Neurosciences, UMCG (Dr. B.J. Eggen). 70-80% confluent cells were subcultured and plated in 6 and/or 24-well plates at a density of 20,000 – 50,000 cells/well for relevant experiments as described below.

Reagents

The following reagents were used: dopamine (H8502, Sigma, USA); haloperidol (Haldol, Janssen-Cilag BV, Tilburg); fluphenazine (Bristol-Myers Squibb, Utrecht); vanoxerine (HY 13217, MedChemexpress LLC); 6-benz-cyclic adeno monophosphate (6-b-cAMP; BioLOG, Bremen, Germany); forskolin (F-9929, LB Laboratories, MA, USA); neutral red (NR; Sigma, USA).

Hypothermia-rewarming induction & treatment regimen

Twenty-four hours after seeding, the BV2 cells were exposed to 24 h hypothermia (4-6 °C) followed by 2 h rewarming at 37 °C, before initiating the viability assay. Dopamine treatment (3 - 700 µM) was initiated 1 h prior to the hypothermic exposure and continued during the whole procedure including the rewarming phase. Pretreatment with respective antagonists (fluphenazine, 0.3 and 10 µM; vanoxerine, 0.3 and 10 µM; haldol, 0.3 and 10 µM) was initiated 20 min prior to incubation with dopamine.

Cell viability assay

Cell viability was assessed by neutral red (NR) assay. Upon completion of the 2 h rewarming phase, the medium was removed and cells were washed with PBS and

incubated at 37 °C for 3 h with NR medium prepared by diluting NR stock (4 mg/ml) solution in DMEM to attain a concentration of 50 µg/ml. Subsequently, the cells were washed with PBS and NR desorb (prepared by dissolving 98 % glacial acetic acid (100 µl), 96 % ethanol (5 ml) in distilled water to result in a 10 ml solution) to dissolve the NR crystals formed during the acidification process. After incubating at room temperature for 10 min on a shaker, the reaction mixture was assessed by measurement of absorbance using a microplate reader at 540 nm.

Western blotting

BV2 cells were harvested after giving PBS washes in 150µl RIPA buffer from 6-well plates, containing protease inhibitor cocktail, sodium orthovanadate, sodium fluoride and β-mercaptoethanol (all from Sigma Aldrich, The Netherlands). The cell lysate was then sheared using insulin syringe and incubated on ice, before determining the protein concentrations using a Biorad assay kit, as per the manufacturer's guidelines (Bio-Rad, Germany). Samples were boiled for 5 min before loading onto the pre-casted SDS-polyacrylamide gel. The gel electrophoresis was carried out at 100 V for 70 min using 30 µg of total protein for each sample. Proteins were subsequently blotted onto nitrocellulose membranes using a transfer buffer solution containing 0.25 mM Tris (pH 8.5), 192 mM glycine and 10% v/v methanol at 4 °C for 60 min at 0.3mA. Thereafter, the membranes were blocked in 5% w/v skimmed milk dissolved in TBST buffer (containing 50mM Tris-HCl, pH 6.8, 150mM NaCl, 0,05% v/v Tween-20). Subsequently, the membranes were incubated overnight at 4 °C with the following antibodies diluted in TBST solution containing 5% BSA (w/v): anti-CBS (sc-271886, Santa Cruz), anti-3MST (HPA 001240, Sigma). After overnight incubation, the membranes were washed 3 times in TBST buffer and incubated with HRP-linked polyclonal secondary antibodies (Dako, Denmark) in 5% BSA-TBST solution for 1 h. Blots were developed using the Western Lighting Ultra substrates (Perkin Elmer Inc., USA) according to the manufacturer's guidelines. Protein bands were visualized using the Gene Genome system (Westburg BV, The Netherlands) and intensities quantified using Gene Tools software.

Statistics

Data are expressed as mean ± SD. Two-tailed t-test was used in comparisons for cell viability as well as for CBS and 3-MST levels between normothermic versus hypothermia-rewarmed cells. One-way ANOVA analysis was used followed by posthoc analysis using Tukey's test for comparisons with treatment groups. A $p<0.05$ was considered to be statistically significant.

RESULTS

Hypothermia-rewarming alters BV2 cellular morphology & compromises viability

Control BV2 cells incubated at 37 °C are shown in figure 1A. Twenty-four hours of hypothermia followed by 2 hours of rewarming resulted in marked detachment of cells with signs of cell death, including bleb formation, nuclear condensation, and cellular fragmentation (Fig. 1B). Further, neutral red assay documented hypothermia and rewarming to significantly reduce cell viability when compared to normothermic control cells (Fig. 1C).

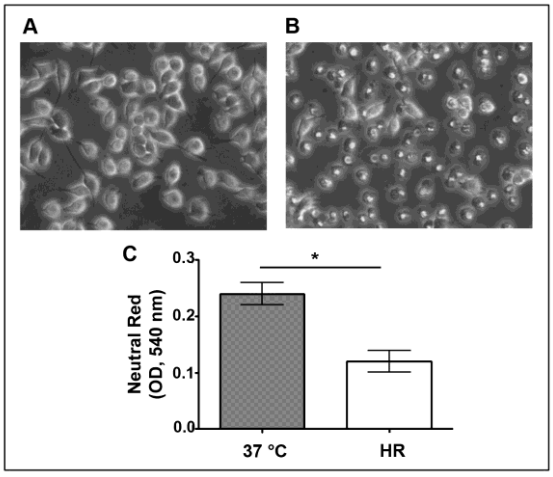

Figure 1: Morphology and viability of BV2 cells following hypothermia and rewarming. **(A)** normothermia, and **(B)** hypothermia (24h) -rewarming injury (2h). **(C)** Spectrophotometric analysis of cell viability using neutral red assay. *p<0.05 normothermic (37 ^{0}C) vs hypothermia-rewarmed cells (HR). Values are represented as mean ± SD.

Dopamine rescues BV2 cells from hypothermia-rewarming injury

The protective effect of dopamine against cooling and rewarming injury was investigated by generating concentration-response curves (Fig. 2). At 3 µM, dopamine did not improve cell survival. Increasing concentrations of dopamine (10, 30, 50 and 100 µM) instituted a significant rescuing effect (p<0.05 versus untreated cells). However, further increases in dopamine concentration (300 and 700 µM) were associated with a decrease in the cell survival. Thus, dopamine displayed a bell-shaped concentration-response curve on cell survival (Fig. 2).

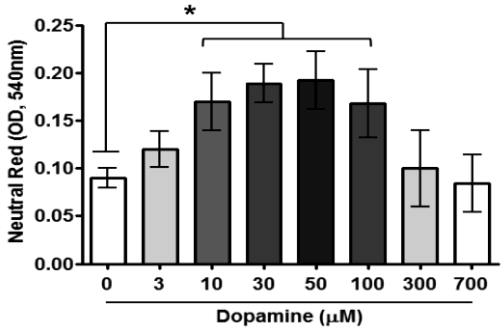

Figure 2: Bell-shaped concentration-response curve of dopamine to protect from cell death. *p<0.05 untreated vs dopamine-treated hypothermia-rewarmed BV2 cells. Values are represented as mean ± SD.

Induction of cAMP mimics the protective effect of dopamine

Since dopamine via its D1/D2 receptor types may affect the cAMP signaling cascade, we set out to investigate involvement of this cascade in dopamine's improvement of cell viability in hypothermia-rewarming injury. Stimulation of cAMP signaling by activation of adenylyl cyclase (forskolin 0.1-100 μM) rescued BV2 cells from hypothermia-rewarming induced cell death in a concentration-dependent manner (Fig. 3A). Comparable results were obtained after administration of a cAMP analogue (6-benz-cAMP, 0.1-100 μM) (Fig. 3B).

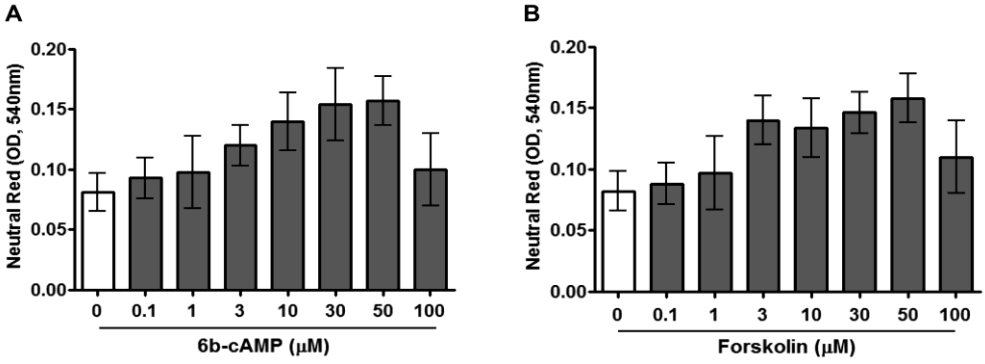

Figure 3: Concentration-dependent effect of 6-benz-cyclicAMP (6b-cAMP**; A)** and forskolin **(B)** on cell viability in hypothermia-rewarmed BV2 cells. Values are represented as mean ± SD.

Rescuing effect of dopamine from hypothermia-rewarming injury is partially attenuated by receptor blockade, but not affected by its reuptake inhibition

To further examine whether dopamine receptor modulation conveys the beneficial effects of dopamine, we next examined the effects of dopamine receptor antagonists (affinity D2>D1: haldol, 0.3 - 100µM; affinity D2~D1: fluphenazine, 0.3 - 10µM) and dopamine re-

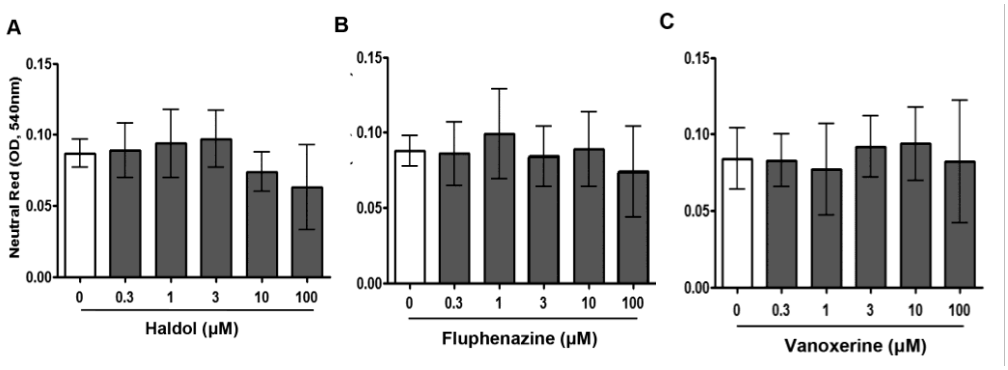

Figure 4: Absence of any effect of haldol **(A)**, fluphenazine **(B)** and vanoxerine **(C)** on cell viability in hypothermia-rewarming injury in BV2 cells. Values are represented as mean ± SD.

uptake blocker (vanoxerine, 0.3 - 10µM). None of these inhibitors influenced BV2 cell viability following hypothermia-rewarming when compared to untreated cells (Fig. 4A-C). Further, both the dopamine receptor antagonist haldol as well as the dopamine re-uptake inhibitor, vanoxerine did not affect the improvement of cell viability by dopamine (Fig. 5A-D). In contrast, pretreatment with the non-selective DR antagonist, fluphenazine, markedly abrogated dopamine's rescuing effect on hypothermia and rewarming induced cell death (Fig. 5 E,F).

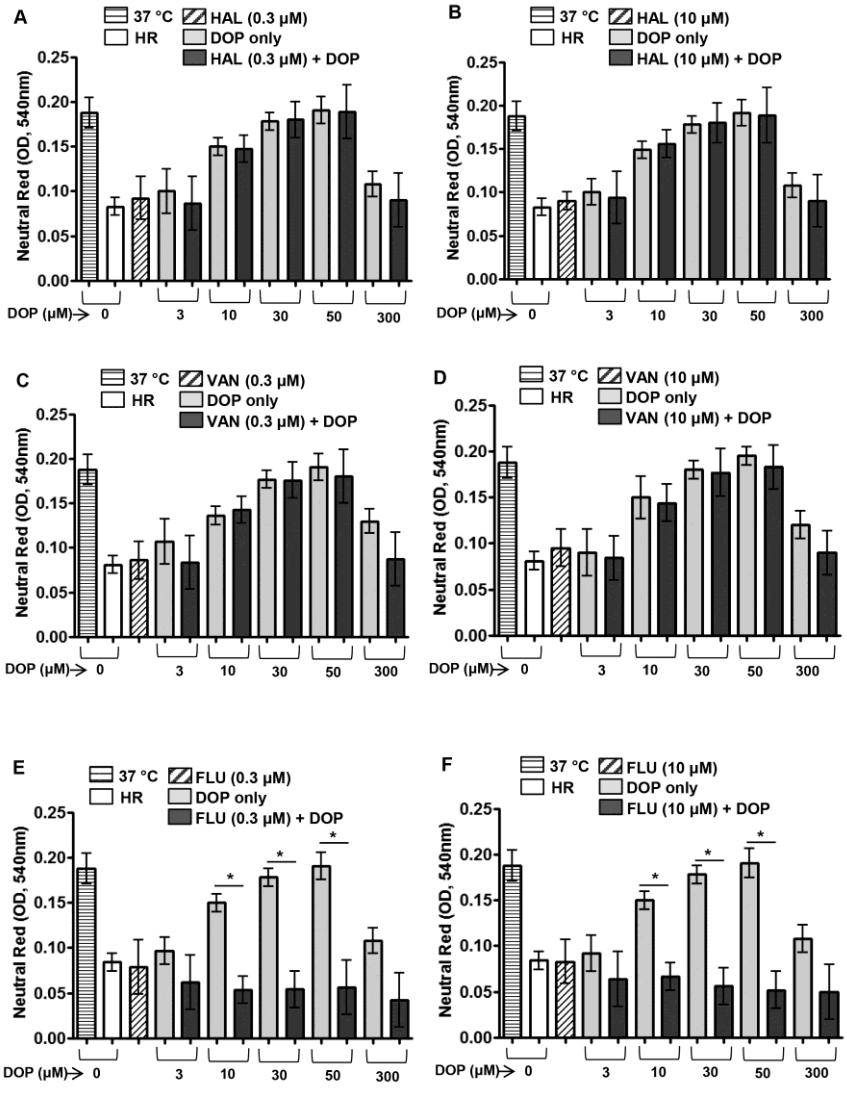

Figure 5: Effect of receptor and reuptake inhibition on dopamine conferred protection in hypothermia-rewarming injury of BV2 cells. Effect of pre-incubation of dopamine with low or high dose of haldol **(A, B),** vanoxerine **(C, D)** and fluphenazine **(E, F)** from hypothermia-rewarming injury on BV2 cell viability. HR, hypothermia-rewarming; HAL. Haldol; DOP, dopamine; VAN, vanoxerine; FLU, fluphenazine. Values are represented as mean ± SD. *p<0.05 dopamine only-treated versus antagonist-treated hypothermia-rewarmed BV2 cells.

Dopamine upregulated CBS and 3-MST levels in hypothermia-rewarmed BV2 cells

As dopamine-induced maintenance of H_2S production in hypothermia and rewarmed smooth muscle cells is associated with upregulation of H_2S synthesizing enzymes (12),

expression of CBS and 3-MST was examined. Hypothermia-rewarming induced a significant downregulation of both CBS and 3-MST levels in BV2 cells ($p<0.05$ versus normothermic control cells, Fig. 6A,B). Incubation with dopamine throughout the procedure induced a concentration dependent increase in both CBS and 3-MST expression, which ultimately normalized CBS expression at 50 µM. Whereas a higher dose of dopamine (300 µM) further upregulated CBS expression (Fig. 6A), it severely downregulated 3-MST levels (Fig. 6B).

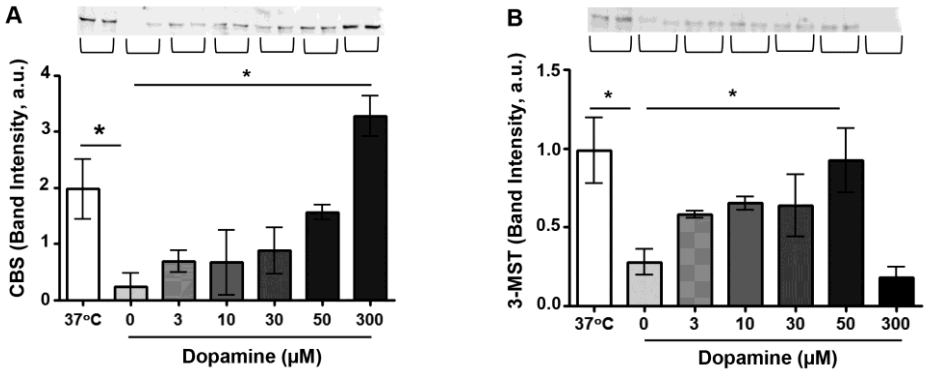

Figure 6: Dopamine effects on CBS and 3-MST expression in hypothermia-rewarmed BV2 cells. Downregulation of CBS (A) and 3-MST (B) expression level is evident upon hypothermia-rewarming injury when compared to normothermic cells, which is alleviated by dopamine treatment in a concentration-dependent manner. Pairs of lanes indicated on Western blots represent successive conditions represented on the X-axis. *$p<0.05$. Values are represented as mean ± SD.

DISCUSSION

In this study, we demonstrated that exogenously administered dopamine improves the viability of BV2 cells undergoing hypothermia-rewarming injury. Dopamine protection seems predominantly mediated by D1-type receptor mediated cAMP production, in view of the actions of cAMP analogues and receptor antagonists, respectively. Further, in contrast to previous reports in cultured smooth muscle arterial cells (12), re-uptake of dopamine does not seem to be involved. Although its mechanism in BV2 cells seems receptor mediated rather than dependent on re-uptake as found previously (12), dopamine treatment was also found to upregulate CBS and 3-MST levels.

Our results implicate a receptor-mediated rescuing effect of dopamine against hypothermia-rewarming injury in BV2 cells, which is accompanied by the preservation of the expression of key H_2S synthesizing enzymes and is mimicked by cAMP analogues. In this context dopamine, apart from its action on α1- and β1-adrenoreceptors, can also bind to 2 major receptor subtypes including the D1 and D2-type of receptors. The D1 subtype

includes D1 and D5 receptors while the D2 family comprise of D2, D3 and D4 (19) receptors. The activation of D1-type of receptors is coupled to G-protein Gsα activating adenyl cyclase and thereby increasing the level of cAMP. In contrast, D2-type receptors coupled to Giα activation inhibit formation of cAMP via inhibiting adenyl cyclase. An increased level of cAMP as evident after D1-type receptor activation can activate several targets such as protein kinase A (PKA), exchange proteins activated by cAMP (EPAC) and cyclic nucleotide gated ion channels.

Our observation contradicts those reported by Yard et al (2004), who suggested dopamine to act receptor independently. In their study, dopamine-treated HUVECs were subjected to 24 h cold preservation injury, and the protection by dopamine appeared to be due to a ROS scavenging effect, possibly related to induction of heme oxygenase-1 expression. Moreover, the protective effect of dopamine did not appear to be mediated by β-receptor activation, and it was unaffected by D1 and/or D2 receptor blockade (17). Instead, dopamine protection was mediated by an oxidative mechanism as addition of antioxidants such as ascorbic acid or N-acetylcysteine blocked the protective effect of dopamine. In our previous study, Talaei et al (2011) observed protection by dopamine of rat smooth muscle aortic cells (SMAC) from hypothermia induced cell death. This protective effect was abrogated by the dopamine reuptake inhibitor, vanoxerine. In the current study, protective action of dopamine was to a large extent abrogated by fluphenazine (D1~D2) but not with haldol (D2>D1) or dopamine reuptake inhibition. Moreover, similar to a D1 receptor-mediated effect via Gsα downstream signalling, receptor independent induction of cAMP rescued the cells, indicating that D1 type is the major dopamine receptor subtype expressed in the BV2 cells. Indeed, mouse microglial cells primarily express receptors of D1-type as shown by Faber et al (2005). Together, our results are consistent with the view that dopamine protection in this study is conveyed by its D1 receptor subtype.

In accord with our previous studies (12,15), dopamine treatment in our study was shown to normalize the levels of CBS and 3-MST in hypothermia-rewarmed BV2 cells when compared to untreated cells. In CNS, CBS is one of the key H_2S synthesizing enzymes primarily in glial cells while 3-MST is considered to be expressed mainly in neurons. Taken together, the D1 receptor mediated action and its downstream targets and the interplay with H_2S synthesizing enzymes could further imply a central protective mechanism in determining the extent of hypothermia-rewarming injury in microglial cells and warrants further exploration.

Dopamine and its related compounds (e.g. dobutamine) have been used in hypotensive episodes (shock) associated with conditions such as myocardial infarction, trauma, septicaemia and renal failure (21,22). Besides, dopamine is one of the most abundant

neurotransmitters in the CNS with a considerable amount found in peripheral tissues as well. With the current ongoing trials to study neuroprotection of hypothermia, the role of dopamine as a potential therapeutic agent in mitigating hypothermia-rewarming associated adverse effects (both central and peripheral) warrants further pre-clinical exploration. Moreover, the underlying mechanisms shown in our model needs further detailed study of the dopamine signalling cascade, including downstream receptor targets involved in cAMP activation and their effects on the regulation of H_2S producing enzymes and H_2S production.

CONCLUSION

Dopamine was shown to rescue BV2 microglial cells against hypothermia-rewarming injury by a receptor-mediated action (mainly D1 type), independent of its reuptake. Besides, the induction of cAMP rescuing the cells against this insult further strengthens the protection via D1–type of receptors and warrants detailed studies exploring downstream targets. Furthermore, dopamine treatment was shown to upregulate levels of H_2S synthesizing enzymes, mainly CBS and 3-MST, following hypothermia and rewarming as observed in our previous studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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